The Patent Office

INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

PRIORITY DOCUMENT

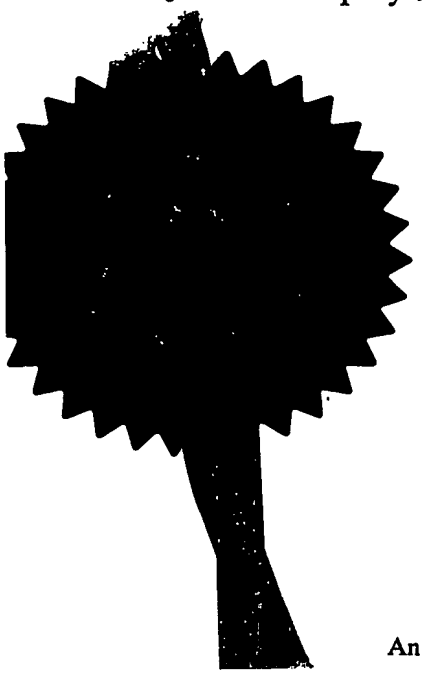
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.



Signed P. Mahoney

Dated 27 January 2004

Patents Form 1/77

Patents Act 1977
(Rule [redacted])

THE PATENT OFFICE
17 JAN 2003
RECEIVED BY HAND

The Patent Office
PATENTS • DESIGNS • TRADE MARKS
COPYRIGHT

20JAN03 E778048-1 000060
P01/7700 0.00-0301124.4

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
South Wales
NP10 8QQ

1. Your reference

AHB/LP6125546

2. Patent application number
(The Patent Office will fill in this part)

0301124.4 17 JAN 2003

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

VERENIGING HET NEDERLANDS
KANKER INSTITUUT
PLESMANLAAN 121
NL-1066 CX AMSTERDAM
THE NETHERLANDS

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

NL

06735030 01

4. Title of the invention

THERAPEUTIC METHODS

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

MEWBURN ELLIS
York House
23 Kingsway
London WC2B 6HP

Patents ADP number *(if you know it)*

109006 ✓

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

YES

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	0
Description	22
Claim *(s)*	2
Abstract	1
Drawing *(s)*	4 + 4

10. If you are also filing any of the following, state how many against each item.

Priority documents	0
Translations of priority documents	0
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	0
Request for preliminary examination and search *(Patents Form 9/77)*	0
Request for substantive examination *(Patents Form 10/77)*	0
Any other documents *(please specify)*	0

11. I/We request the grant of a patent on the basis of this application.

Signature [signature] Date 17 JANUARY 2003

12. Name and daytime telephone number of person to contact in the United Kingdom

ADRIAN H. BRASNETT
020 7240 4405

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 08459 500505.

b) Write your answers in capital letters using black ink or you may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

Therapeutic Methods

Field of the Invention.

The present invention relates to the treatment of cylindromatosis and more generally to the modulation of other conditions associated with activation of the transcription factor NF-κB, such as inflammation.

Background to the Invention.

Cylindromas are rare benign adnexal tumours that arise primarily on the scalp in humans. They occur at any age but usually appear in early adulthood. There are 2 distinct clinical forms: a solitary form, which is sporadic, and a multiple form, which is dominantly inherited, referred to as familial cylindromatosis. The lesions are pink-red, nodular, firm, and usually painless, and vary in size from several millimetres to more than 6 cm in diameter. The tumours grow slowly in size and number throughout life; in severe cases, they may cover the entire scalp and are known as "turban tumours." At present, treatment is by surgery to remove the tumours followed by reconstruction of the affected area.

The condition has been linked to the chromosomal region 16q12-13 and more recently Bignell, G. R. et al., (*Nat Genet* 25, 160-5 (2000)) identified the gene, *CYLD*, in this region which is mutated in individuals with cylindromatosis. The gene is regarded as a tumour suppressor gene though its mode of action has not been elucidated.

Nuclear factor κB (NF-κB) is a sequence-specific transcription factor that is known to be involved in the inflammatory and innate immune responses. NF-κB is activated by release from an inhibitory factor, referred to as IkB. NF-κB is a heterodimer consisting of a 50 kDa (p50) and a 65 kDa (p65) DNA-binding

subunit. NF-κB contributes to the so-called "immediate-early" activation of defence genes if cells are exposed to primary or secondary pathogenic stimuli.

It has also been found that NF-κB and the signalling pathways that are involved in its activation are also important for tumour development, in that NF-κB also regulates cell proliferation and apoptosis. NF-κB has been shown to be constitutively activated in several types of cancer cell.

Disclosure of the Invention.

The present inventors have identified a regulatory pathway present in cells which directly links the action of CYLD to the suppression of NF-κB. Loss of CYLD thus leads to an increase in NF-κB activity, which in turn causes an increase in anti-apoptopic gene function. This may result in the disruption of the balance of pro- and anti-apoptopic gene regulation in cells of the skin, leading to the growth of the benign tumours associated with cylindromatosis.

Thus in one aspect, the invention provides a method of treating an individual with cylindromatosis by administering to the individual an effective amount of an NF-κB inhibitor.

In another aspect, the invention provides the use of an NF-κB inhibitor for the manufacture of a medicament for the treatment of cylindromatosis. Alternatively, the invention provides an NF-κB inhibitor for use in a method of treatment of cylindromatosis.

In a further aspect, the finding that the action of CYLD is to suppress NF-κB activity provides a new target for the treatment of diseases associated with activation of NF-κB. Thus the invention provides a method of treating such a disease in an

individual by administering to the individual an effective amount of an agent which increases expression of CYLD. In another aspect, the invention provides the use of an agent which increases expression of CYLD for the manufacture of a medicament for the treatment of a disease associated with activation of NF-κB. Alternatively, the invention provides an agent which increases expression of CYLD for use in a method of treatment of a disease associated with activation of NF-κB.

In a further aspect, the finding that NF-κB activity can be regulated by CYLD provides a novel assay method for the development of new agents for human or animal therapy. Thus the invention provides an assay method which includes the steps of:

providing a cell culture in which CYLD activity is suppressed or missing;

bringing the culture into contact with an agent to be assayed; and

determining the effect of the agent on the activity of NF-κB.

These and other aspects of the invention are described further herein below.

Description of the Drawings.

Figure 1 Genome-wide DUB knockdown screen.

a, Overview of identified DUBs using the Ensembl database, and construction of the knockdown library. Four independent 19-mer targeting sequences were selected per predicted DUB mRNA transcript. Annealed oligonucleotides were cloned separately into pSUPER and four DNA preparations were pooled per DUB to yield a knockdown library with a complexity of 50 DUBs. **b**, 293 cells were co-transfected as indicated and immunoblotted with a GFP antibody, p21-RFP served as a transfection control. **c**, U2-OS cells were co-transfected with a NF-κB luciferase reporter

construct (3 x RE) and individual members of the DUB knockdown library. After 48 hrs, cells were stimulated overnight with TNF-α (20 ng/ml) and luciferase activity was measured. SV40 Renilla luciferase served as an internal control. **d**, U2-OS cells were transfected with HA-tagged CYLD and pSUPER-CYLD or empty vector. Whole cell extracts were immunoblotted with a HA antibody. GFP served as a transfection control.

Figure 2 CYLD is an antagonist of NF-κB signalling. **a**, U2-OS cells were transfected with a NF-κB luciferase reporter and pSUPER-CYLD or empty vector. Forty-eight hours after transfection cells were stimulated overnight with PMA (200 nM) or TNF-α (20 ng/ml) and luciferase activity was measured. SV40 Renilla luciferase served as an internal control. **b**, 293 cells were transfected as indicated, lysates were prepared 48 hours later, and protein complexes were immunoprecipitated (IP) using Flag antibody. IPs were immunoblotted for HA-tagged CYLD (upper panel), whole cell extracts were immunoblotted for Flag-tagged IκBα, IKKβ and NEMO/IKKγ (lower panel) and for HA-tagged CYLD (middle panel). **c**, U2-OS cells were co-transfected with Flag-tagged IKKβ and pSUPER-CYLD or empty vector. Cells were stimulated as indicated, IKKβ was immunoprecipated from cell lysates and incubated with GST-IκBα (1-72) in the presence of ^{32}P-γATP (upper panel). Immunoprecipitated IKKβ was visualized by immunoblotting with Flag antibody (lower panel). **d**, U2-OS cells were electroporated with pSUPER-CYLD or empty vector, together with a puromycin resistance marker. Transfected cells were selected for 48 hours with puromycin (2.0 μg/ml) and stimulated with TNF-α (15 ng/ml) as indicated. Whole cell extracts were immunoblotted for endogenous IκBα.

Figure 3 CYLD loss protects against TNF-α induced apoptosis. **a**, **b**, Hela cells were efficiently electroporated as indicated. Prior to inducing apoptosis with TNF-α (10 ng/ml) and

cyclohexamide (10 µg/ml) cells were pre-treated with PMA (200 ng/ml) for 2 hours or left untreated. Photos were taken 16 hours after TNF-α/cyclohexamide addition (3a) and percentage of viable cells was quantified using trypan blue exclusion method (3b). **c**, Hela cells were transfected with a NF-κB luciferase reporter and pSUPER-CYLD or empty vector. Seventy-two hrs after transfection cells were stimulated with PMA (200 nM) for two hours and luciferase activity was measured.

Figure 4 Anti-apoptotic effects of CYLD loss can be reversed by aspirin.

a, b, U2-OS cells were transfected with a NF-κB luciferase reporter and pSUPER-CYLD or empty vector. Forty-eight hours after transfection cells were left untreated, stimulated with PMA (200 nM) or PMA and aspirin (4a, 10 mM) or PMA and prostaglandin A1 (4b, 8 µM). Luciferase activity was measured the next day. **c**, Hela cells were efficiently electroporated as indicated. After 72 hours cells were pre-treated with PMA (200 ng/ml) or PMA and aspirin (8 mM) for two hours. Apoptosis was induced by addition of TNF-α (10 ng/ml) and cyclohexamide (10 µg/ml). Photos were taken 16 hours later. **d**, Simple model for the antagonistic action of IKK inhibitors and CYLD loss on cell survival.

Detailed Description of the Invention.

As indicated above, individuals with cylindromatosis are those with a lesion in the *CYLD* gene located on chromosome 16q12-13 leading to a mutation or lack of expression of the CYLD gene product. Bignell *et al*, *ibid*, report the identification of the structure of *CYLD* and report that many affected individuals have mutations located in the 3′ two-thirds of the *CYLD* coding sequence. Other human individuals may have deletions of the entire region of the chromosome where the gene is located.

individuals with cylindromatosis can be administered an effective amount of an NF-κB inhibitor for the treatment of their condition.

By "treatment", it is meant any degree of alleviation of the disease including a suppression in the rate of growth of the tumours. This will be beneficial in lengthening the time required before surgical intervention is required.

A number of agents which are known to inhibit NF-κB are known in the art. For example, US 5,985,592 discloses that pentoxifylline or functional derivatives or metabolites thereof can be used for the treatment of diseases characterised by activation of NF-κB. The phrase "pentoxifylline or functional derivatives/metabolites thereof" refers to the compound 1-(5-oxohexyl)-3,7-dimethylxanthine (pentoxifylline), and oxidation-, reduction-, substitution- and/or rearrangement-products thereof, such as, for example, metabolite-1 through metabolite-7 as described by Luke and Rocci in J. Chromatogr. 374(1):191-195 (1986) (e.g., 1-(5-hydroxyhexyl)-3,7-dimethyl-xanthine (metabolite-1)), as well as synthetic variants thereof (e.g., propentofylline).

US 6,090,542 teaches that NF-κB activity may be suppressed by treating cells with a substance which inhibits the proteolytic degradation of the alpha subunit of IκB, IκB-α.

Other agents which are known to inhibit NF-κB include aspirin, ibuprofen, sulindac, flurbiprofen and salicylates; and cyclo-pentenone prostaglandins (cyPGs) such as A-type and J-type cyPGs, for example prostaglandin A1 (PGA1) and cyPG 15-deoxy-delta12-14-PGJ2.

A further class of agents comprise nucleic acids including anti-sense nucleic acids, siRNA and ribozymes. These agents may be directed to NF-κB mRNA in target cells in the individual, in order to reduce expression of the gene. The nucleic acids may be delivered as naked DNA or formulations thereof, e.g. liposomal formulations designed to enhance cellular uptake. Gene therapy vectors which express the nucleic acids in the target cells may also be used.

Agents which inhibit NF-κB may be administered to a subject in need of treatment in any suitable form. Usually the agent will be in a form of a pharmaceutical composition in which the agent is mixed with a pharmaceutically acceptable carrier. The carrier will be adapted to be suitable for the desired route of administration of the agent. The agent may be administered, for example, orally, topically, subcutaneously or by other routes.

In general, pharmaceutical compositions contemplated for use in the present invention can in the form of a solid, a solution, an emulsion, a dispersion, a micelle, a liposome, and the like, wherein the resulting composition contains one or more of the active compounds contemplated for use herein, as active ingredients thereof, in admixture with an organic or inorganic carrier or excipient suitable for enteral or parenteral applications. The active ingredients may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, and any other form suitable for use. The carriers which can be used include glucose, lactose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea, medium chain length triglycerides, dextrans, and other carriers suitable for use in manufacturing preparations, in solid, semisolid, or liquid form.

Pharmaceutical compositions containing the active ingredients contemplated herein may be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsions, hard or soft capsules, or syrups or elixirs. Compositions intended for oral use may be prepared according to any method known in the art for the manufacture of pharmaceutical compositions.

In some cases, formulations for oral use may be in the form of hard gelatin capsules wherein the active ingredients are mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate, kaolin, or the like. They may also be in the form of soft gelatin capsules wherein the active ingredients are mixed with water or an oil medium, for example, peanut oil, liquid paraffin, or olive oil.

The pharmaceutical compositions may be in the form of a sterile injectable suspension. This suspension may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides, fatty acids (including oleic acid), naturally occurring vegetable oils like sesame oil, coconut oil, peanut oil, cottonseed oil, etc., or synthetic fatty vehicles like ethyl oleate or the like. Buffers, preservatives, antioxidants, and the like can be incorporated as required.

For example, formulations of compounds for topical administration include transdermal formulations designed to enhance uptake of the active agent through the skin. Transdermal delivery devices, e.g. patches, are well known in the art and may be used to present a transdermal formulation of the agent. For example, US 6,368,618 describes a formulation suitable for the transdermal administration of aspirin comprising aspirin (e.g. in an amount 1% to about 30% w/w) together with at least one alcohol (e.g. in an amount 1% to about 40% w/w) selected from the group consisting of isopropyl alcohol, ethyl alcohol and propylene glycol; and at least one melting point depressing agent selected from the group consisting of thymol, menthol, eucalyptol, eugenol, methyl salicylate, phenyl salicylate, capsaicin, butylated hydroxytoluene, a local anesthetic agent and any combination thereof, said melting point depressing agent present in the composition in an amount of less than about 1/4 (e.g. from 1/20 to 1/4) of the weight of the aspirin; said composition having spontaneously equilibrated aqueous and oil phases, wherein the aspirin is in substantially melted form at 25°C, and wherein the concentration of the aspirin in the oil phase is, by weight, at least about 40% of the weight of the oil phase.

The amount of agent administered will be dependent upon the nature of the agent and its route and dose of administration, and taking into account the patient and their particular needs. For example, aspirin and other NSAIDs administered orally can be provided in a unit dosage form of from 100 to 1000 mg, to be taken 1 to 5 times a day. Other routes of administration of the same drug may be dosed to an equivalent level. Reference may be made to US 5,985,592 for doses of pentoxifylline or functional derivatives or metabolites thereof. Prostaglandins may be administered in the range of 0.1 to 100 mg/kg body weight per day.

It is possible that CYLD may also be mutated in other cancers, such as breast, lung, colon and prostate cancers. Thus the agents and their formulations and routes and doses of delivery referred to herein may be used in the treatment of other cancer conditions associated with a mutation in the *CYLD* gene.

The role of CYLD in suppressing the anti-apoptopic effects of NF-κB provides the potential to treat diseases associated with cellular proliferation by enhancing the levels of CYLD in a cell in order to suppress the release of NF-κB from IκB. Such diseases include interstitial lung disease, human fibrotic lung disease (e.g., idiopathic pulmonary fibrosis (IPF), adult respiratory distress syndrome (ARDS), tumor stroma in lung disease, systemic sclerosis, Hermansky-Pudlak syndrome (HPS), coal worker's pneumoconiosis (CWP), chronic pulmonary hypertension, AIDS associated pulmonary hypertension, and the like), human kidney disease (e.g., nephrotic syndrome, Alport's syndrome, HIV-associated nephropathy, polycystic kidney disease, Fabry's disease, diabetic nephropathy, and the like), glomerular nephritis, nephritis associated with systemic lupus, liver fibrosis, myocardial fibrosis, pulmonary fibrosis, Grave's ophthalmopathy, drug induced ergotism, cardiovascular disease, cancer, Alzheimer's disease, scarring, scleroderma, glioblastoma in Li-Fraumeni syndrome, sporadic glioblastoma, myeloid leukemia, acute myelogenous leukemia, myelodysplastic syndrome, myeloproliferative syndrome, cancers such as breast, lung, colon, prostate or gynecological cancer (e.g., ovarian cancer, Lynch syndrome, and the like), Kaposi's sarcoma, Hansen's disease, inflammatory bowel disease, and the like.

Elevating the levels of CYLD may be achieved by administration of an agent which enhances the production of native CYLD in the cell, or by introduction of a gene therapy vector designed to express CYLD in target cells. Gene therapy of somatic cells can

be accomplished by using, e.g., retroviral vectors, other viral vectors, or by non-viral gene transfer (for clarity cf. T. Friedmann, Science 244 (1989) 1275; Morgan 1993, RAC DATA MANAGEMENT REPORT, June 1993).

Vector systems suitable for gene therapy are, for instance, retroviruses (Mulligan, R. C. (1991) in Nobel Symposium 8: Ethiology of human disease at the DNA level (Lindsten, J. and Pattersun Editors), pages 143-189, Raven Press), adeno associated virus (McLughlin, J. Virol. 62 (1988), 1963), vaccinia virus (Moss et al., Ann. Rev. Immunol. 5 (1987) 305), bovine papilloma virus (Rasmussen et al., Methods Enzymol. 139 (1987) 642) or viruses from the group of the herpes viruses such as Epstein Barr virus (Margolskee et al., Mol. Cell. Biol. 8 (1988) 2937) or Herpes simplex virus.

There are also known non-viral delivery systems. See for example US 6,228,844 (Wolff). For this, usually "nude" nucleic acid, preferably DNA, is used, or nucleic acid together with an auxiliary such as, e.g., transfer reagents (liposomes, dendromers, polylysine-transferrine-conjugates (Wagner, 1990; Felgner et al., Proc. Natl. Acad. Sci. USA 84 (1987) 7413)).

Gene therapy vectors comprising a sequence encoding CYLD (the sequence of which is available in Bignell *et al*, *ibid*, operably linked to a promoter functional in the target cells may thus be used to suppress the anti-apoptopic effects of NF-κB. Promoters suitable for use in various vertebrate systems are well known. For example, strong promoters include RSV LTR, MPSV LTR, SV40 IEP, and metallothionein promoter. The CMV IEP may be more preferable for human use.

Assays according to the invention may be performed in any cell line, preferably a mammalian cell line, more preferably a human

cell line, in which the NF-κB pathway is active. The cells will either naturally contain deficient CYLD (e.g. by originating from a subject with cylindromatosis) or may be modified to suppress, either temporally or permanently, the *CYLD* gene. Suppression of the activity of *CYLD* may be achieved by siRNA, as illustrated in the accompanying examples.

In assays of the invention, a cell culture in which CYLD activity is suppressed or missing will be brought into contact with an agent to be assayed. Following incubation of the cells, for example from 1 to 48 hours, the activity of NF-κB will be determined.

The amount of an agent which may be added to an assay of the invention will normally be determined by trial and error depending upon the type of compound used. Typically, from about 0.01 to 100 nM concentrations of agent compounds may be used, for example from 0.1 to 10 nM. Agent compounds which may be used may be natural or synthetic chemical compounds used in drug screening programmes. Extracts of plants or microorganisms which contain several characterised or uncharacterised components may also be used.

The activity of NF-κB may be determined by a variety of means. For example, the amount of NF-κB protein in a cell may be examined by immunological techniques, such as western blotting. The amount of NF-κB RNA in the cell may be examined, using for example northern blotting or quantitative PCR. Alternatively, the amount of NF-κB can be examined using a reporter gene assay, i.e. by determining the amount of expression of a reporter gene (e.g. firefly luciferase, secreted alkaline phosphatase (SEAP) or green fluorescent protein) whose promoter comprises one or more (e.g. two, three or four) tandem copies of the k enhancer

element Such constructs are commercially available (e.g. the pNF-κB-Luc vector from BD Biosciences Clontech, Palo Alto, CA).

The following examples illustrate the invention.

Examples

Protein ubiquitination is used primarily to target proteins for proteasome-mediated destruction[1]. Protein ubiquitination is a dynamic process that involves large families of ubiquitin-conjugating enzymes and ubiquitin ligases that add ubiquitin molecules to substrates and a less-studied family of deubiquitinating enzymes (DUBs) that remove ubiquitin from protein substrates. Two classes of DUBs can be distinguished: ubiquitin C-terminal hydrolases (UCHs) and ubiquitin-specific processing proteases (UBPs)[1-3]. The UBP enzymes remove ubiquitin residues from large substrates by cleaving at the C-terminus of the ubiquitin moiety and are candidate antagonists of the ubiquitin conjugation/ligation system. A role for DUB genes in cancer is suggested by the fact that this family contains both oncogenes[6,7] and tumour suppressor genes[4]. In addition, members of the DUB family have been described to interact with p53[8] and BRCA1[9] and the von Hippel Lindau (VHL) tumour suppressor gene[10].

The strategy we pursued to study the function of the individual members of this family of DUB enzymes was to inhibit the expression of independent family members through RNA interference and search for phenotypes induced by loss of DUB expression. We first searched several nucleotide sequence databases for genes with homology to the catalytic domain of DUBs. A total of 50 genes could be identified harbouring this motif, including the cylindromatosis tumour suppressor gene (CYLD)[4] and the TRE2 oncogene[6] (Fig. 1a). Next, we retrieved the cDNA sequences corresponding to these potential DUBs and selected four unique 19-mer sequences from each transcript for

cloning into pSUPER, a vector that mediates suppression of gene expression through the synthesis of short hairpin RNAs having siRNA-like properties[11]. We chose to make four knockdown vectors against each DUB to increase the chance that a significant inhibition of DUB expression would be obtained. In total, we made 200 knockdown vectors, which were subsequently pooled into 50 sets of 4 vectors, where each set of vectors was designed to target a single DUB transcript (Fig, 1a).

To ask how effective the set of four knockdown vectors inhibited DUB gene expression, we fused the open reading frame of four of the DUBs to GFP and determined the levels of GFP-DUB fusion protein expression in the absence and presence of co-expression of the DUB knockdown vectors. Fig. 1b shows that a significant reduction in protein levels was induced by all four DUB knockdown vectors, whereas control p21-RFP fusion protein was unaffected. We conclude that this strategy allows efficient inhibition of DUB expression.

To begin to study the function of the members of the DUB family, we asked if suppression of any of the DUBs could affect the activity of NF-κB, a cancer-relevant transcription factor with marked anti apoptotic activity[12]. We transfected an NF-κB-luciferase reporter gene, together with each of the 50 sets of 4 DUB knockdown vectors into human U2-OS cells and measured the effect of DUB knockdown on Tumour Necrosis Factor-α (TNF-α)-activated levels of NF-κB. Fig. 1c shows that only one of the sets of DUB knockdown vectors (#36) significantly enhanced TNF-α-activation of NF-κB. This becomes particularly apparent when the 50 reporter values are sorted by increasing activity (Fig. 1c, lower panel). This effect was specific, as knockdown of DUB#36 did not affect an E2F-luciferase reporter or a Hypoxia Induced Factor 1-α (HIF-1α)-responsive promoter (data not shown). Importantly, the DUB#36 set of knockdown vectors targets

the cylindromatosis tumour suppressor gene CYLD[4], a confirmed de-ubiquitinating enzyme[13], suggesting that CYLD is a regulator of NF-κB.

To ask if the CYLD knockdown vectors efficiently suppress abundance of the CYLD protein, we generated a HA-epitope-tagged CYLD expression vector and co-transfected this vector with the pSUPER-CYLD knockdown vector, the most active of the four CYLD knockdown vectors in the initial pool of four CYLD knockdown vectors. Fig.1d shows that HA-CYLD protein levels were significantly reduced by pSUPER-CYLD, confirming that CYLD is efficiently targeted for suppression by the CYLD knockdown vector.

NF-κB is held in an inactive form in the cytoplasm by IκB inhibitor proteins. Signalling through the IκB kinase (IKK) complex, containing the IκB kinases IKKα and β and the structural component NEMO (or IKKγ), causes phosphorylation and subsequent degradation of IκB, allowing nuclear translocation of NF-κB[12,14]. In principle, the observed effect of CYLD knockdown on TNF-α stimulation of NF-κB could result from an effect of CYLD on the TNF-α receptor, a more downstream effect on the IKK complex or directly on the IκB/NF-κB complex itself. Since the tumour promoter phorbol 12-myristate 13-acetate (PMA) activates NF-κB downstream of the TNF-α receptor, we asked if CYLD knockdown also affected PMA-mediated activation of NF-κB. Fig. 2a shows that CYLD knockdown did not enhance basal level of NF-κB activity, but further increased both PMA and TNF-α activated NF-κB levels. This suggests that CYLD loss affects NF-κB downstream of the TNFα receptor.

Next, we asked if CYLD could physically associate with known members of the NF-κB signalling machinery. Fig. 2b shows that CYLD co-immunoprecipitated specifically with NEMO/IKKγ, but not

with IκBα or IKKβ. This suggests that CYLD acts on the IκB kinase complex through direct association. To address this, we measured IKKβ kinase activity following TNF-α stimulation in the presence and absence of CYLD knockdown, using an *in vitro* kinase assay. Fig. 2c shows that in the absence of TNF-α, no IKKβ kinase activity towards IκBα could be detected. As expected, TNF-α treatment significantly stimulated IKKβ kinase activity. Importantly, this activity was further enhanced when cells were co-transfected with CYLD knockdown vector (compare Fig 2c, lanes 5, 6). No effects were seen of CYLD knockdown on IKKβ protein levels (Fig. 2c, lower panel) suggesting that CYLD does not act to regulate IKKβ abundance. Consistent with an increase in IKKβ kinase activity by CYLD knockdown, we observed that CYLD knockdown resulted in a more significant reduction in IκBα levels, an endogenous substrate of IKKβ kinase (Fig. 2D). Together, these data indicate that CYLD acts as an antagonist of the IKK complex through direct binding to the non-catalytic NEMO/IKKγ component and that reduction of CYLD expression stimulates signalling through the IKK complex.

When combined with inhibitors of transcription or translation, TNF-α is a potent inducer of apoptosis in certain cell types. This pro-apoptotic activity of TNF-α can be inhibited by simultaneous activation of NF-κB, which activates a number of anti-apoptotic genes[15]. Since CYLD knockdown stimulates PMA-induced activation of NF-κB, we asked if CYLD and PMA also collaborate to inhibit TNF-α induced apoptosis. To address this, we treated Hela cells with TNF-α in the presence of cyclohexamide (CHX) to induce apoptosis both with and without pre-treatment with PMA (see methods). Fig. 3a and 3b show that a 12-hour treatment with TNF-α efficiently induced apoptosis in some 95% of the Hela cells. As expected, pre-treatment with PMA resulted in an approximately four-fold increase of the number of viable cells. Significantly, PMA pre-treatment in Hela cells

that had been transfected with CYLD knockdown vector resulted in an even larger fraction of surviving cells, suggesting that loss of the CYLD tumour suppressor gene confers resistance to induction of apoptosis (Fig. 3a, b), most likely through activation of NF-κB. Consistent with this notion, CYLD knockdown and PMA also collaborated in NF-κB activation in Hela cells (Fig. 3c).

NF-κB can be inhibited by a number of pharmacological agents, including aspirin and prostaglandin A1 (PGA1)[5,16]. Both compounds have been shown to act on IKKβ, the same kinase that is hyper-activated as a result of CYLD knockdown (Fig. 2c). This raises the possibility that the effect of CYLD knockdown on NF-κB activation can be counteracted by aspirin or PGA1. To address this, we transfected U2-OS cells with the NF-κB-luciferase reporter plasmid and activated NF-κB 48 hours after transfection with PMA. As was observed before, knockdown of CYLD further enhanced PMA-stimulated activation of NF-κB (Fig. 4a, b). Strikingly, this effect of CYLD knockdown on NF-κB activity could be significantly suppressed both by aspirin and by PGA1 (Fig. 4a, b), indicating that these compounds can compensate for CYLD suppression in this assay.

As was discussed above, it is possible that loss of the CYLD tumour suppressor gene confers resistance to apoptosis through activation of NF-κB. If this notion is correct, one would expect that the protective effect of CYLD knockdown on apoptosis can be reversed by the NF-κB inhibitor aspirin. We tested this by treating Hela cells with TNF-α and PMA in the presence of CYLD knockdown. Fig. 4c shows that the combination of CYLD knockdown and PMA treatment again conferred significant resistance to TNF-α-induced apoptosis (upper right panel). Significantly, exposure of cells to 10mM aspirin prior to TNF-α treatment completely abolished the protective effect of CYLD knockdown on TNF-α-

induced apoptosis (Fig. 4c) indicating that aspirin can also reverse the anti-apoptotic effects of CYLD loss. This result is consistent with the notion that CYLD knockdown protects from TNF-α-induced apoptosis through activation of IKKβ and subsequently of NF-κB.

We describe here the first high-throughput RNA interference screen in mammalian cells to identify novel regulators of NF-κB. We focused on ubiquitin-specific processing proteases (UBPs) as these proteins are potential antagonists of the well-studied ubiquitin conjugating enzymes and ubiquitin ligases. Unexpectedly, we identify the familial cylindromatosis tumour suppressor gene (*CYLD*) as a novel negative regulator of NF-κB, thus establishing the first direct link between the NF-κB signalling cascade and a tumour suppressor gene. Our results provide an explanation for the deregulated proliferation of the epidermal appendices in patients with familial mutations in the *CYLD* gene (Fig. 4d). It is well-established that NF-κB is required for normal skin proliferation[14]. For instance, mice with suppressed NF-κB have defects in the development of hair follicles and exocrine glands, resulting from increased rates of apoptosis[17]. Furthermore, female NEMO/IKKγ heterozygous mice have severe skin defects, including increased apoptosis in keratinocytes[18]. A similar skin defect is found in the human genetic disorder incontinentia pigmenti (IP), which also results from mutations in the NEMO/IKKγ gene[19]. Thus, inhibition of NF-κB in the skin causes an increase in apoptosis. We therefore suggest that the deregulated proliferation in the skin appendices in patients suffering from cylindromatosis results from a perturbation in the balance between proliferation and apoptosis in favor of proliferation, resulting from an increase in active NF-κB. That cylindromas result from a relatively mild perturbation of normal proliferation is also supported by the notion that most cylindromas have a diploid karyotype and are

rarely metastatic[4]. The observation that the enhanced protection from apoptosis that results from *CYLD* suppression can be reversed by simple pharmacological agents like aspirin and prostaglandin A1 suggests a strategy to restore normal growth control in patients suffering from familial cylindromatosis.

Methods.

Materials, Antibodies, and Plasmids Construction. To generate DUB knockdown vectors, four annealed sets of oligonucleotides encoding short hairpin transcripts corresponding to one DUB enzyme were cloned individually into pSUPER. Bacterial colonies were pooled and used for plasmid preparation. To generate GFP-DUB fusion proteins the corresponding DUB enzymes were PCR amplified using DNA from human cDNA libraries as a template and cloned into pEGFP-N1. pNF-κB-Luc vector was obtained from Clontech, SV40-Renilla from Promega. PMA, TNF-α and Prostaglandin A1 and cyclohexamide were purchased from Sigma. HA-tagged CYLD was PCR amplified from human cDNA libraries and cloned into pCDNA 3.1 (-), Flag tagged NEMO was generated by cloning an EcoRI-XbaI NEMO containing fragment into pcDNA-flag. Anti-IκB-α (c-21) and HA tag (Y-11) antibodies were obtained from Santa Cruz, anti-Flag M2 from Sigma and anti-GFP rabbit polyclonal serum was kindly provided by J. Neefjes.

Cell cultures, transient transfections and reporter assays. All cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. High efficiency electroporation of cells was done as described[20]. Reporter assays were carried out using calcium-phosphate transfection of 0.5 μg NF-κB-Luc, 1 ng SV40-Renilla and 2.5 μg pSUPER vectors. Forty-eight hours after transfection cells were stimulated with 200nM PMA or 20 ng/ml TNF-α and luciferase activity was measured 72 hrs post-transfection. Sodium acetylsalicylic acid (10 mM) or

Prostaglandin A1 (8 μM) was added to the cells 48 hrs after transfection, and reporter activity was measured 72 hrs after transfection. In Hela cells NF-κB activity was measured 2 hours after PMA stimulation.

Immunoblotting, immunoprecipitation and kinase assay.
Western blots were performed using whole cell extracts, separated on 8-12% SDS-PAGE gels and transferred to polyvinylidine difuoride membranes (Millipore). Western blots were probed with the indicated antibodies. Transformed human embryonic kidney cells (293 cells) were transfected by calcium-phosphate precipitation with the indicated plasmids, 48 hrs post-transfection cells were lysed in ELB buffer (0.25M NaCL, 0.1% NP-40, 50 mM Hepes pH 7.3) supplemented with "Complete" protease inhibitors (Roche), centrifuged and protein complexes were immunoprecipitated with 2 μg of the indicated antibodies conjugated to protein G sepharose beads. The beads were washed four times with ELB buffer and protein complexes were eluted by boiling in SDS-sample buffer and resolved on 10% SDS-PAGE. Imunoprecipitation/kinase assays were performed essentially as described[21].

Apoptosis Assays.
Electroporated Hela cells with the indicated plasmids were treated with 200 nM PMA for 2-3 hrs 72 hrs post-transfection followed by 12 hrs incubation in medium containing 10 ng/ml TNF-α and 10 μg/ml cyclohexamide. Viable cells were quantified using the trypan-blue exclusion method. Alternatively, apoptotic cells were removed by PBS washing, adherent cells were fixed in 4% paraformaldehyde and stained using 0.1% crystal violet (Sigma) and the optical density at 590 nm was determined as described[22]. To inhibit NF-κB activity medium was supplemented with 10mM Sodium acetylsalicylic acid 3.5 hrs before TNF-α addition.

References.

1. Wilkinson, K. D. Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome. *Semin Cell Dev Biol* **11**, 141-8. (2000).

2. Chung, C. H. & Baek, S. H. Deubiquitinating enzymes: their diversity and emerging roles. *Biochem Biophys Res Commun* **266**, 633-40. (1999).

3. D'Andrea, A. & Pellman, D. Deubiquitinating enzymes: a new class of biological regulators. *Crit Rev Biochem Mol Biol* **33**, 337-52. (1998).

4. Bignell, G. R. et al. Identification of the familial cylindromatosis tumour-suppressor gene. *Nat Genet* **25**, 160-5. (2000).

5. Yin, M. J., Yamamoto, Y. & Gaynor, R. B. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. *Nature* **396**, 77-80. (1998).

6. Nakamura, T., Hillova, J., Mariage-Samson, R. & Hill, M. Molecular cloning of a novel oncogene generated by DNA recombination during transfection. *Oncogene Res* **2**, 357-70. (1988).

7. Papa, F. R. & Hochstrasser, M. The yeast DOA4 gene encodes a deubiquitinating enzyme related to a product of the human tre-2 oncogene. *Nature* **366**, 313-9. (1993).

8. Li, M. et al. Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. *Nature* **416**, 648-53. (2002).

9. Jensen, D. E. et al. BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression. *Oncogene* **16**, 1097-112. (1998).

10. Li, Z. et al. Ubiquitination of a novel deubiquitinating enzyme requires direct binding to von Hippel-Lindau tumor suppressor protein. *J Biol Chem* **277**, 4656-62. (2002).

11. Brummelkamp, T. R., Bernards, R. & Agami, R. A System for Stable Expression of Short Interfering RNAs in Mammalian Cells. *Science* **296**, 550-553. (2002).

12. Karin, M., Cao, Y., Greten, F. R. & Li, Z. W. NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* **2**, 301-10. (2002).

13. Borodovsky, A. et al. Chemistry-based functional proteomics reveals novel members of the deubiquitinating enzyme family. *Chem Biol* **9**, 1149-59. (2002).

14. Smahi, A. et al. The NF-kappaB signalling pathway in human diseases: from incontinentia pigmenti to ectodermal dysplasias and immune-deficiency syndromes. *Hum Mol Genet* **11**, 2371-5. (2002).

15. Baud, V. & Karin, M. Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol* **11**, 372-7. (2001).

16. Rossi, A. et al. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IkappaB kinase. *Nature* **403**, 103-8. (2000).

17. Schmidt-Ullrich, R. et al. NF-kappaB activity in transgenic mice: developmental regulation and tissue specificity. *Development* **122**, 2117-28. (1996).

18. Schmidt-Supprian, M. et al. NEMO/IKK gamma-deficient mice model incontinentia pigmenti. *Mol Cell* **5**, 981-92. (2000).

19. Smahi, A. et al. Genomic rearrangement in NEMO impairs NF-kappaB activation and is a cause of incontinentia pigmenti. The International Incontinentia Pigmenti (IP) Consortium. *Nature* **405**, 466-72. (2000).

20. Agami, R. & Bernards, R. Distinct initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage. *Cell* **102**, 55-66 (2000).

21. Chen, G., Cao, P. & Goeddel, D. V. TNF-induced recruitment and activation of the IKK complex require Cdc37 and Hsp90. *Mol Cell* **9**, 401-10. (2002).

22. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. & Lowe, S. W. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88**, 593-602 (1997).

CLAIMS:

1. A method of treating an individual with cylindromatosis by administering to the individual an effective amount of an NF-κB inhibitor.

2. The method of claim 1 wherein said inhibitor is aspirin or prostaglandin A1.

3. Use of an NF-κB inhibitor for the manufacture of a medicament for the treatment of cylindromatosis.

4. Use according to claim 3 wherein said inhibitor is aspirin or prostaglandin A1.

5. A method of treating a diseases associated with activation of NF-κB which in an individual comprises administering to an individual an effective amount of an agent which increases expression of CYLD.

6. Use of an agent which increases expression of CYLD for the manufacture of a medicament for the treatment of a disease associated with activation of NF-κB.

7. An assay method which includes the steps of:

providing a cell culture in which CYLD activity is suppressed or missing;

bringing the culture into contact with an agent to be assayed; and

determining the effect of the agent on the activity of NF-κB.

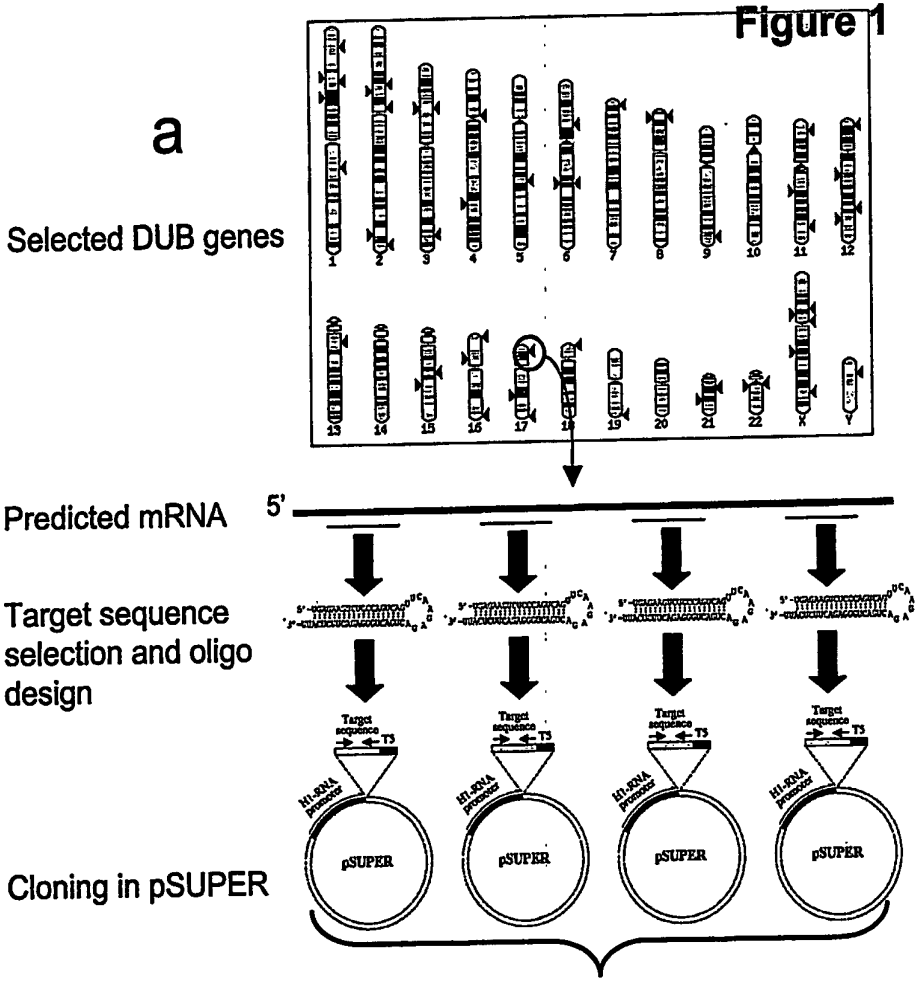
8. The method of claim 8 wherein CYLD activity is suppressed using siRNA.

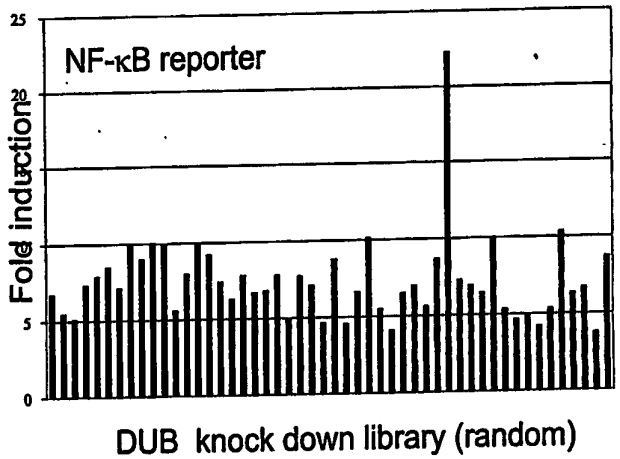
9. The method of claim 7 or 8 wherein the effect of the agent is determined using a reporter gene construct.

Abstract

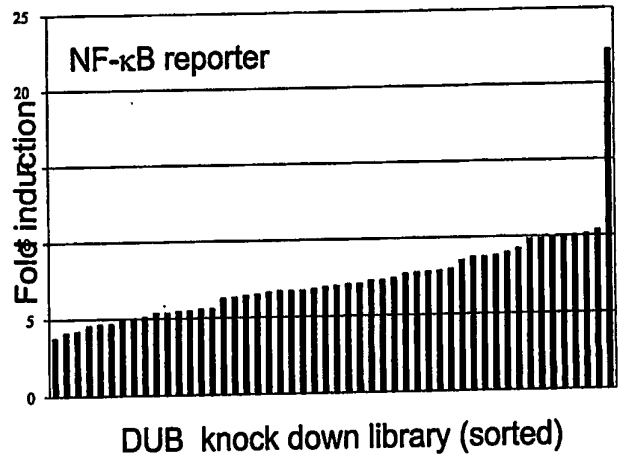
Ubiquitin-proteasome-mediated proteolysis plays a major part in many biological processes, including cell cycle and apoptosis[1]. The enzymes that mediate ubiquitin-conjugation have been studied intensively, but much less is known about the ubiquitin-specific proteases that mediate de-ubiquitination of cellular substrates[2,3]. To study this gene family, we constructed a collection RNA interference vectors that were designed to suppress one of fifty human de-ubiquitinating enzymes (DUBs). We report here that inhibition of the familial cylindromatosis tumor suppressor gene (*CYLD*)[4], having no known function, enhances activation of the anti-apoptotic transcription factor NF-κB. We show that CYLD binds to the NEMO/IKKγ component of the IκB kinase complex and regulates its activity. Importantly, we find that inhibition of *CYLD* expression increases resistance to apoptosis. This suggests that deregulated proliferation, which results from loss of the *CYLD* tumour suppressor gene, may result from enhanced resistance to apoptosis. We show that the anti-apoptotic effect of *CYLD* repression can be relieved by the NF-κB inhibitor aspirin[5], which suggests a therapeutic intervention strategy to reverse the deregulated growth control in patients suffering from familial cylindromatosis.

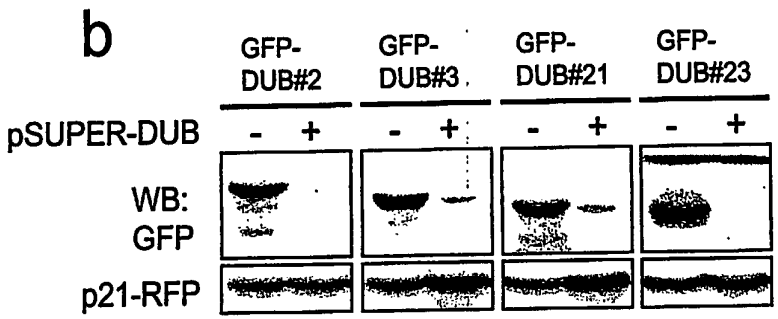
Figure 1


a
Selected DUB genes
Predicted mRNA
5'
Target sequence selection and oligo design
Cloning in pSUPER
Target sequence
T3
H1-RNA promoter
pSUPER
DUB knock down library


NF-κB reporter
Fold induction
25
20
5
0
DUB knock down library (random)

c


NF-κB reporter
Fold induction
25
20
5
0
DUB knock down library (sorted)


b
GFP-DUB#2
GFP-DUB#3
GFP-DUB#21
GFP-DUB#23
pSUPER-DUB
- + - + - + - +
WB: GFP
p21-RFP

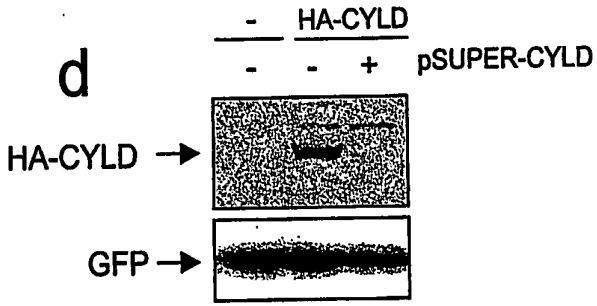
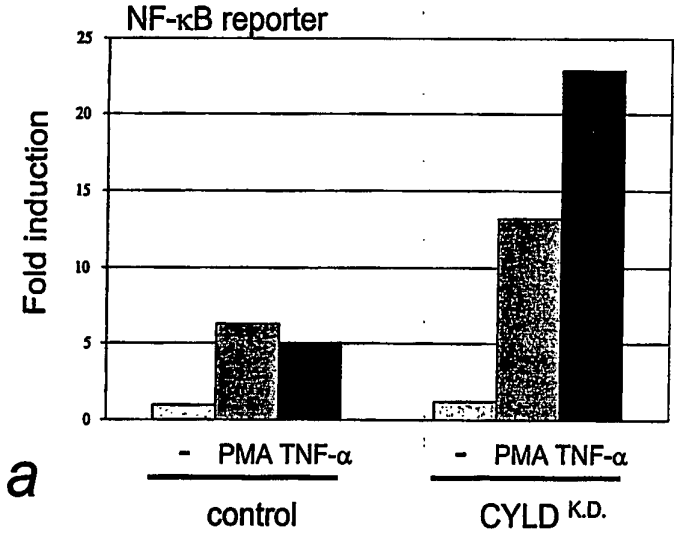
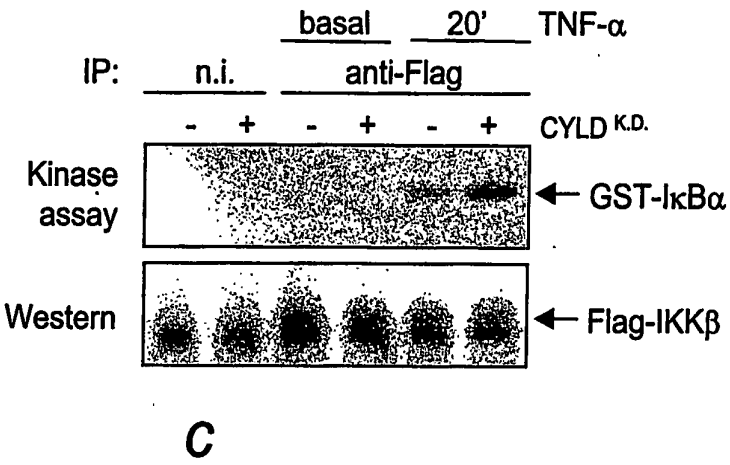
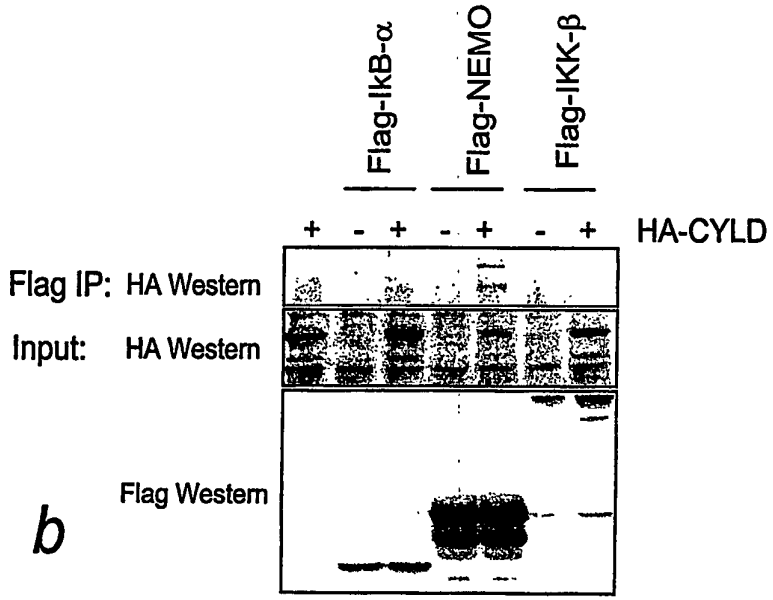

d
- HA-CYLD
- - + pSUPER-CYLD
HA-CYLD
GFP

Figure 2


NF-κB reporter
Fold induction
25
20
15
10
5
0
- PMA TNF-α
- PMA TNF-α
control
CYLD K.D.
a


basal
20'
TNF-α
IP:
n.i.
anti-Flag
- + - + - +
CYLD K.D.
Kinase assay
GST-IκBα
Western
Flag-IKKβ
c


Flag-IkB-α
Flag-NEMO
Flag-IKK-β
+ - + - + - + HA-CYLD
Flag IP: HA Western
Input: HA Western
Flag Western
b

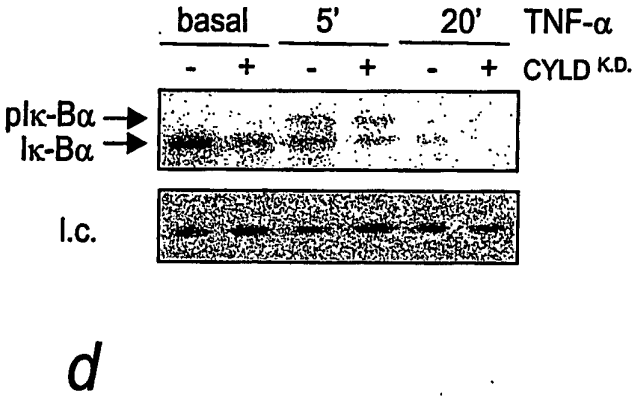

basal
5'
20'
TNF-α
- + - + - + CYLD K.D.
pIκ-Bα
Iκ-Bα
l.c.
d

Figure 3

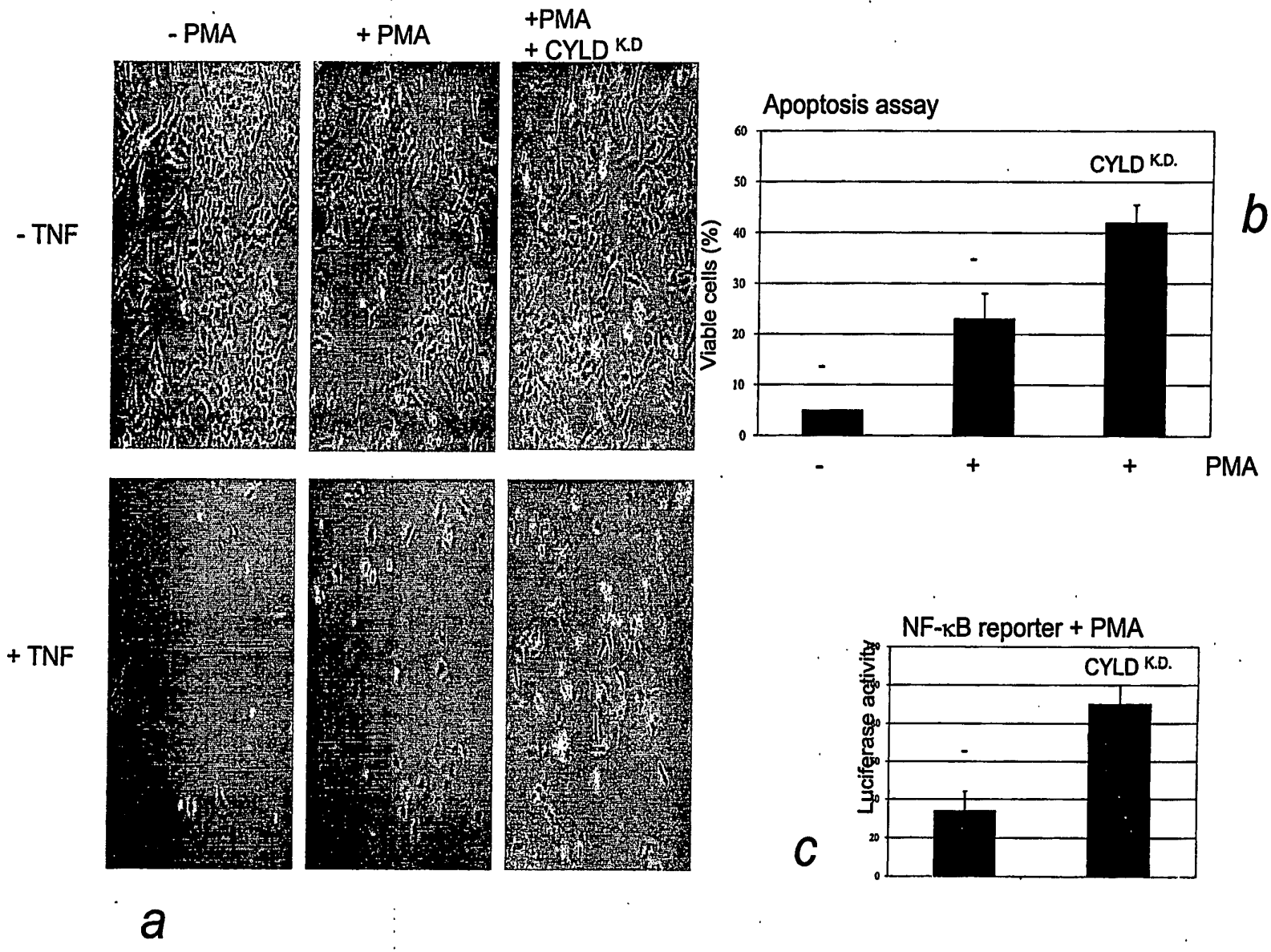
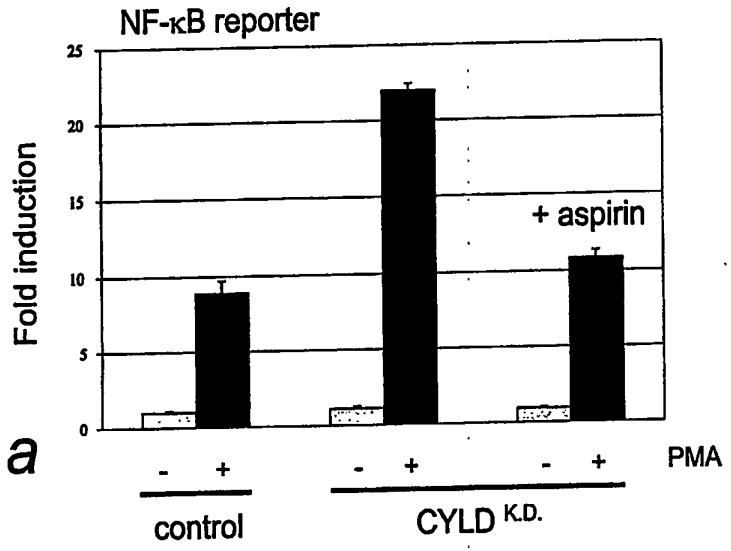
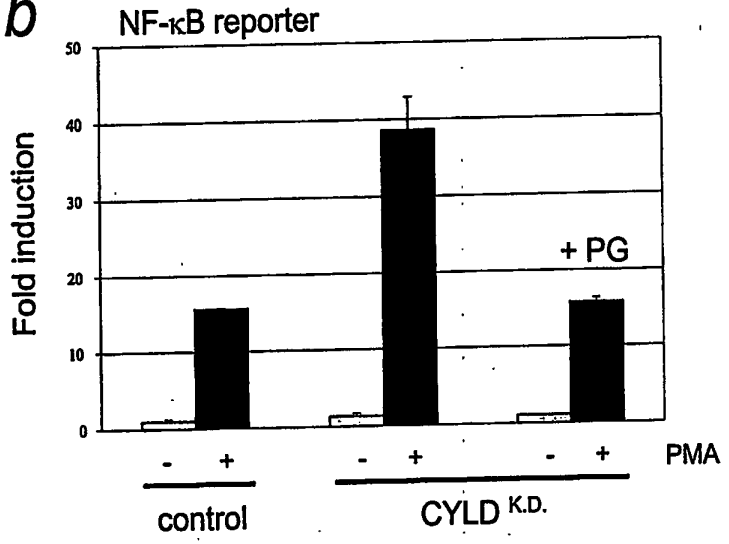

- PMA
+ PMA
+PMA
+ CYLD K.D
- TNF
+ TNF
a
Apoptosis assay
CYLD K.D.
Viable cells (%)
60
50
40
30
20
10
0
-
+
+
PMA
b
NF-κB reporter + PMA
CYLD K.D.
Luciferase activity
c

Figure 4

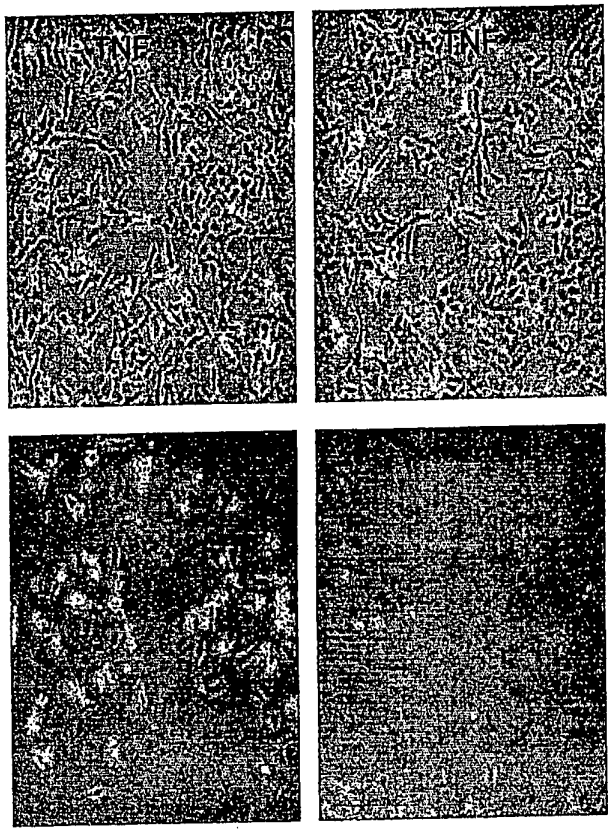


a

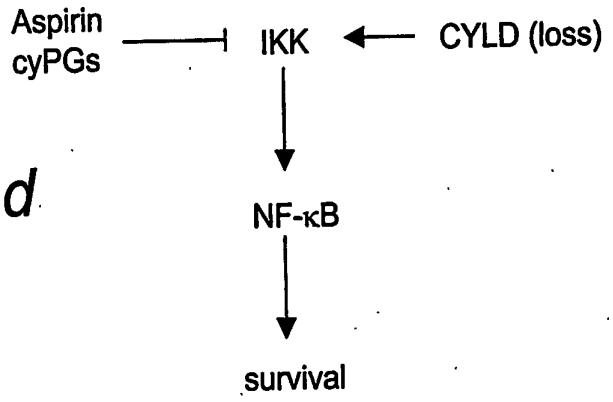


b

c



d



PCT Application

PCT/EP2004/000408